

PRELIMINARY AMENDMENT

Applicant(s): Stice

Serial No.: 09/809,662

Confirmation No.: 5744

Filed: March 15, 2001

For: EFFECTIVE NUCLEAR REPROGRAMMING IN MAMMALS



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Please replace the paragraph at page 2, lines 3-8 with the following rewritten paragraph.

Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

Q2 Citations: Campbell et al., *Nature*, 380, 64 (1996); Cheong et al., *Biol. Reprod.*, 48, 958 (1993); Cibelli et al., *Science*, 280, 1256 (1998); Kato et al., *Science*, 282, 2095 (1998); Onishi et al., *Science*, 289, 1188 (2000); Prather et al., *Biol. Reprod.*, 37, 859 (1987); Polejaeva et al., *Nature*, 407, 86 (2000); Prather et al., *Biol. Reprod.*, 41, 414 (1989); Stice et al., *Biol. Reprod.*, 39, 657 (1988); Wakayama et al., *Nature*, 394, 369 (1998); Willadsen, *Nature*, 320, 63 (1986); and Wilmut et al., *Nature*, 385, 810 (1997).

Please replace the paragraph at page 3, lines 14-19 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

Q3 Improvements in oocyte activation in various species have been vigorously pursued (reviewed in Prather et al., *Theriogen.*, 51, 498 (1999)). Progress has been made by increasing calcium and/or decreasing protein phosphorylation in the oocyte (mice, Szöllösi et al., *J. Cell Sci.*, 104, 861 (1993); cattle, Susko-Parrish et al., *Dev. Biol.*, 166, 729 (1994) and Susko-Parrish et al., (U.S. Patent 5,496,720)).

Please replace the paragraph at page 15, line 24 to page 16, line 9 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

*Enucleation*

Oocytes may be enucleated before introduction of donor genetic material.

Q4 Enucleation of oocytes may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm, or by chemical treatment (see, for instance, Baguisi et al., *Theriol.*, 53, 209 (2000)). If enucleation is performed prior to introduction of donor genetic material, it may be conducted using methods previously described for enucleating MII oocytes (Tao et al., *Anim. Reprod. Sci.*, 56, 133-41 (1999)) or by methods such as described by

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Goto et al., (*Anim. Sci. J.*, 70, 243-245 (1999)). The oocytes may then be screened to identify those successfully enucleated. This screening can be done by staining the oocytes with a detectable marker that specifically binds to DNA (for instance, 1 µg/ml 33342 Hoechst dye in HEPES buffered hamster embryo culture medium (HECM, Seshagiri et al., *Biol. Reprod.*, 40, 599-606, (1989)), and then viewing under ultraviolet irradiation for less than 10 seconds either the oocytes or the cytoplasm and maternal genetic material removed during the enucleation procedure. The oocytes that have been successfully enucleated can then be placed in a suitable culture medium, e.g., TCM-199, G1/G2, or CR1aa plus 10% serum (Stice et al., U.S. Patent 5,945,577).

Please replace the paragraph at page 16 line 15 to page 17, line 3 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

as  
If the oocyte used to produce the NT embryo was not enucleated, then the NT embryo can be enucleated. Within the NT embryo, the maternal genetic material can be distinguished from the donor genetic material by, for instance, the position of the donor nucleus within the NT embryo, formation of the first polar body, or a combination thereof. The known location of the donor genetic material within the NT embryo is based on where it was placed in the perivitelline space in relation to the location of the maternal genetic material. The maternal genetic material is near the opening placed in the zona pellucida during transfer of the donor genetic material, preferably the donor genetic material is placed away from that area. Therefore that area of cytoplasm (near the opening in the zona) can be removed via either enucleation pipette or by expulsion of cytoplasm through the opening in the zona, preferably by enucleation pipette (see, e.g., Prather et al., *Biol. Reprod.*, 37, 859 (1987); and Goto et al., *Anim. Sci. J.*, 70, 243-245 (1999)). With regard to the second method, in some cases the oocyte may progress in

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meiosis to MII after introduction of the donor genetic material. If so, then the first polar body can also be used as landmark to find the maternal genetic material. Hoechst dye can be used to visualize genetic material, including confirming the presence of the maternal genetic material in the removed cytoplasm. These methods may be used alone or in conjunction with each other to verify location of chromosomes and verify enucleation of the oocyte.

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Please replace the paragraph at page 18, lines 14-31 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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Bovine oocytes and NT embryos may be activated by the method of Yang et al. (*Biol. Reprod.*, 46(Suppl 1), 117 (1992)), more preferably, by exposing bovine oocytes to about 1  $\mu$ M to about 100  $\mu$ M ionomycin, preferably about 50  $\mu$ M ionomycin, for 10 minutes and about 1  $\mu$ g/ml to about 100  $\mu$ g/ml cycloheximide, preferably about 10  $\mu$ g/ml cycloheximide, for about 2 hours to about 10 hours, preferably about 6 hours. Preferably, bovine oocytes and NT embryos are activated by exposure to agents that increase the level of cations in the cell, followed by exposure to agents that decrease protein synthesis in the cell and/or agents that are microfilament inhibitors. Most preferably, bovine oocytes and NT embryos are exposed to about 1  $\mu$ M to about 100  $\mu$ M calcium ionophore, preferably about 5  $\mu$ M calcium ionophore, for about 10 minutes. This is followed by incubation in about 1  $\mu$ g/ml to about 10  $\mu$ g/ml cytochalasin B, preferably about 5  $\mu$ g/ml cytochalasin B, and about 1  $\mu$ g/ml to about 100  $\mu$ g/ml cycloheximide, preferably about 10  $\mu$ g/ml cycloheximide, for about 1 hour. This is followed by incubation in about 1  $\mu$ g/ml to about 100  $\mu$ g/ml cycloheximide, preferably about 10  $\mu$ g/ml cycloheximide, for about 5 hours. Preferably, after the activation treatments, bovine NT embryos are cultured in BARC medium (Powell et al., *Theriogen.*, 55, 287 (2001)).

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Please replace the paragraph at page 19, line 31 to page 20, line 16 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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An activated NT embryo may be transferred immediately into a recipient animal or cultured for up to about 8 days in, for instance, KSOM medium, NCSU-23 medium, BARC medium, G1.2/G2.2 culture medium, or others well known to the art (see for instance Stice et al., U.S. Patent 5,945,577; Wells et al., *Biol. Reprod.*, 60, 996-1005 (1999); and Tao et al., *Anim. Reprod. Sci.*, 56, 133-41 (1999)). Preferably, an activated NT embryo is cultured for between about 12 hours to about 36 hours (for porcine NT embryos) or for about 7 to about 8 days (for bovine NT embryos). Then, intact NT embryos (some cleaved) are transferred into a synchronous recipient animal, i.e., the transferred NT embryo is at the same stage, or about a day before or a day after, as a fertilized embryo would be in the recipient. For pigs, from about one to about 300 NT embryos can be transferred into each recipient female but typically about 50 to about 150 embryos are transferred and ideally 100 embryos are transferred. Methods of surgical and non-surgical transfer in animals is well known in the art. For instance, surgical and non-surgical transfer in pigs is described by Curnock et al., (*Amer. J. Vet. Res.*, 37, 97-98 (1976)), and Hazeleger et al., (*Theriogenol.*, 51, 81-90 (1999)). Preferably, the animal is of the same species as the donor genetic material of the NT embryo.

Please replace the paragraph at page 30, lines 4-17 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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Fusion was performed in a 100 mm dish filled with 15 ml of Zimmermann fusion medium (Zimmermann et al., *J. Membrane Biol.*, 67, 165-182 (1982)). Two stainless steel wires (100  $\mu$ m diameter) attached to micromanipulators were

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used as electrodes. The single cell-oocyte complex was sandwiched between the electrodes and oriented with the contact surface between the enucleated oocyte and the donor cell perpendicular to the electrodes. The distance between the electrodes was about 100  $\mu$ m. Membrane fusion was induced by applying a single direct current pulse of 250 V/mm for a duration of 20  $\mu$ seconds with a prepulse of alternating current field of 5 V, 1 MHz for 2 seconds using an LF 101 Fusion Machine (TR Tech Co., Tokyo, Japan). Following the fusion, the NT embryos were washed in G1.2 medium (Vitrolife, Inc., Englewood Colorado) (Gandhi et al., *Mol. Reprod. Dev.*, 58, 269-275 (2001)) and cultured for a period of 1 hour in 100  $\mu$ l of the same medium. Fusion was then determined by microscopic examination.

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con

Please replace the paragraph at page 34, line 30 to page 35, line 10 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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When transferred into recipient animals, the NT embryos are cultured for between about 12 hours to about 36 hours and then intact NT embryos (some cleaved) are transferred into a synchronous recipient gilt or sow. One to 300 NT embryos can be transferred into each recipient female but typically about 50 to about 150 embryos are transferred and ideally about 100 embryos are transferred. Methods of surgical and non-surgical transfer in pigs are well known in the art (Hazeleger et al., (*Theriogenol.*, 51, 81-90 (1999))). Ultrasound and non-return to estrus are used to determine which recipients are pregnant. NT fetuses if needed for tissue or cell transplantation can be harvested during the pregnancy through surgical recovery. If live pigs are desired the pregnancy last approximately 114 days and some pigs may require neonatal assistance in the form of oxygen supplementation and other interventions (Hill et al., *Theriogenol.*, 51, 1451 (1999)).

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Please replace the paragraph at page 37, line 23 to page 38, line 13 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

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Oocyte preparation

G10  
*In vitro* maturation of bovine immature oocytes and enucleation were performed as described previously (Cibelli et al., *Science*, 280, 1256 (1998); Wells et al., *Biol. Reprod.*, 60, 996-1005 (1999); and Kubota et al., *Proc. Natl. Acad. Sci. USA*, 97, 990-995 (2000)). Briefly, bovine cumulus-oocyte complexes (COCs) were recovered by aspiration of small antral follicles on ovaries obtained from a slaughterhouse. Only COCs with a compact, nonatretic cumulus oophorus-corona radiata and a homogenous ooplasm were selected. They were matured in TCM 199 (Gibco Inc, Grand Island, New York) supplemented with 10% FBS, 50 µg/ml sodium pyruvate, 1% (volume:volume) penicillin/streptomycin (10,000 Units/ml penicillin G, 10,000 µg/ml streptomycin), 1 ng/ml rIGF-1 (Sigma), 0.01 Units/ml bLH and 0.01 Units/ml bFSH (Sioux Biochem. Sioux Center, Iowa) in four-well plates overlaid with mineral oil. Maturation was performed at 39°C in a humidified 5% CO<sub>2</sub> in air for 16-18 hours. After maturation, the cumulus-corona was totally removed by vortexing COCs in TL HEPES medium containing 100 Units/ml hyaluronidase (Sigma). Oocytes matured for 16-18 hours were enucleated in MII phase with a 15 µm (internal diameter) glass pipette (Ependorf Munich, Germany) by aspirating the first polar body and MII plate in a small volume of surrounding cytoplasm in TL HEPES supplemented with 7.5 µg/ml Cytochalasin B (Sigma). The oocytes were previously stained in TL HEPES containing 2 µg/ml Hoechst 33342 and 7.5 µg/ml Cytochalasin B for 10-15 minutes. Enucleation was performed under ultraviolet light to ensure removal of oocyte chromatin.

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Please replace the paragraph at page 38, lines 15-26 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

Fusion, activation, and culture of NT units

*CL*  
Oocyte-granulosa NT units were fused by using a needle-type electrode (Miyoshi et al., *Biol. Reprod.*, 62, 1640-1646 (2000); Goto et al., *Anim. Sci. J.*, 70, 243-245 (1999)) in Zimmermann's fusion medium (Zimmermann et al., *J. Membrane Biol.*, 67, 165-182 (1982)). The single cell-oocyte NT unit was sandwiched between two wires arranged in a straight line and attached to micromanipulators. The contact surface between the oocyte and the donor cell was parallel to the electrodes. The distance between the electrodes was approximately 150  $\mu\text{m}$  (the diameter of the oocytes). A single direct current pulse of 40 V for a duration of 20  $\mu\text{seconds}$  was applied. Following the pulse, the complexes were cultured in TCM 199 supplemented with 10 % FBS for 2 hours and fusion rates were determined.

Please replace the paragraph at page 38, line 27 to page 39, line 6 with the following rewritten paragraph. Per 37 C.F.R. §1.121, this paragraph is also shown in Appendix A (attached herewith) with notations to show the changes made.

*A'*  
Activation of NT embryos was performed as described previously (Goto et al., *Anim. Sci. J.*, 70, 243-245 (1999)) after modification. Briefly, 2 hours after fusion, NT embryos were exposed to 5  $\mu\text{M}$  calcium ionophore (free acid, Sigma) for 10 minutes, followed by incubation in TCM 199 supplemented with 10% FBS, 5  $\mu\text{g/ml}$  Cytochalasin B (Sigma), and 10  $\mu\text{g/ml}$  Cycloheximide (Sigma) for 1 hour at 39°C in 5 %  $\text{CO}_2$  in air and in TCM 199 supplemented with 10 % FBS and 10  $\mu\text{g/ml}$  Cycloheximide for 5 hrs at 39°C in 5 %  $\text{CO}_2$ , 5 %  $\text{O}_2$  and 90 %  $\text{N}_2$ . After the activation treatments, NT embryos were cultured in BARC medium (Powell et al., *Theriogen.*, 55, 287 (2001)) in four-well plates overlaid with mineral oil at 39°C in 5 %  $\text{CO}_2$ , 5 %  $\text{O}_2$  and 90 %  $\text{N}_2$  for 7-8 days.